

Analysis of Cannabinoids from Biological Specimens by LC/MS/MS

1 Introduction

Marijuana, obtained from the *Cannabis sativa* plant, is a commonly abused illicit drug. It is typically dried and smoked. Δ^9 -Tetrahydrocannabinol (THC) is the primary psychoactive component of marijuana. 11-hydroxy- Δ^9 -tetrahydrocannabinol (THC-OH) is a major active metabolite of THC. 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH) is a major inactive metabolite of THC.

2 Scope

This procedure is used to qualitatively screen and confirm THC and its primary metabolites in blood specimens. It can also be used to qualitatively screen and identify THC-COOH in urine specimens. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Biological specimens are commonly assayed for the presence of THC, THC-OH, and THC-COOH. Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. Blood specimens are prepared via protein precipitation using acetonitrile. Urine specimens are prepared by hydrolysis with a strong base, followed by neutralization with acid. Prepared samples are extracted using solid phase extraction (SPE). Extracts are analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) in the multiple reaction mode (MRM).

4 Specimens

This procedure uses a biological fluid such as: blood, serum, plasma, or urine. Typically, 0.5 mL of blood or urine is used. Dilution of samples due to limited specimen volume or suspicion of high drug and metabolite concentrations is acceptable.

5 Equipment/Materials/Reagents

- a. Disposable test tubes (silanized glass)
- b. Pipettors with disposable tips

- c. Vortexer
- d. Heating block
- e. Heated evaporator with nitrogen
- f. Glacial acetic acid (ACS grade)
- g. Sodium acetate trihydrate (reagent grade)
- h. 0.1 M Sodium Acetate Buffer (pH 7.0): To a 250-mL volumetric flask, add 3.4 g sodium acetate trihydrate and 200 mL deionized water. Mix well and adjust to $6.5 < \text{pH} < 7.5$ by slow addition of 1 N hydrochloric acid. Bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.
- i. Potassium hydroxide (reagent grade)
- j. 11.8 N Potassium Hydroxide (Hydrolysis Reagent) (KOH): To a 100-mL Nalgene volumetric flask add 66 g potassium hydroxide and 50 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store at room temperature in Nalgene container. Stable 1 year.
- k. Centrifuge
- l. Acetonitrile (Optima grade)
- m. Autosampler vials
- n. CEREX PolyChrom THC solid phase extraction columns (3 or 6 cc)
- o. Ammonium Hydroxide (Reagent grade)
- p. Water:Acetonitrile:Ammonia (90:10:1): Combine 90 mL deionized water, 10 mL acetonitrile and 1 mL ammonium hydroxide and mix well. Prepare fresh.
- q. Hexane (HPLC grade)
- r. Ethyl Acetate (Optima grade)
- s. Acetic Acid (ACS grade)
- t. Hexane:Ethyl Acetate:Acetic Acid (88:10:2): Combine 88 mL hexane, 10 mL ethyl acetate and 2 mL acetic acid and mix well. Prepare fresh.

- u. LC Mobile Phase A (0.1% Formic Acid in Water): Combine 500 mL deionized water and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
- v. LC Mobile Phase B (0.1% Formic Acid in Acetonitrile): Combine 500 mL acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
- w. ABI 6500+ QTRAP Mass Spectrometer equipped with a Shimadzu LC System
- x. Xterra MS C18 (5cm x 3mm x 5µm) analytical column
- y. Positive pressure solid phase extraction manifold
- z. Water (deionized and Optima grade)
- aa. Methanol (Optima grade)
- bb. Formic Acid (Optima LC/MS grade)

6 Standards and Controls

- a. d3-THC Stock Standard (0.1 mg/mL):
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- b. d3-THC-OH Stock Standard (0.1 mg/mL):
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- c. d3-THC-COOH Stock Standard (0.1 mg/mL):
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- d. THC Internal Standard (IS) Working Solution (d3-THC, d3-THC-OH and d3--THC-COOH – 0.25/1.25 µg/mL):
In a 10-mL volumetric flask, combine 0.025 mL each of the d3--THC and d3-THC-OH stock standards. Add 0.125 mL of the d3--THC-COOH stock standard. Dilute to the mark with methanol. Mix well. Store below 0°C. Stable for at least 1 year.
- e. THC Stock Standard (1.0 mg/mL):
Purchased from Cerilliant International and/or Lipomed. Storage conditions and stability determined by manufacturer.

f. THC-OH Working Solution (Soln) (0.1 mg/mL):

Purchased from Cerilliant International and/or Lipomed. Storage conditions and stability determined by manufacturer.

g. THC-COOH Working Soln (0.1 mg/mL):

Purchased from Cerilliant International and/or Lipomed. Storage conditions and stability determined by manufacturer.

h. THC Working Soln (0.1 mg/mL):

Combine 0.1 mL of the THC Stock Standard and 0.9 mL methanol and mix well. Store refrigerated in a crimped vial. Prepare fresh.

i. Cannabinoid High Control (Ctl) Soln (0.5/2.5 µg/mL):

To a 10-mL volumetric flask, add 50 µL the THC and THC-OH Working Solutions and 250 µL of the THC-COOH Working Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.

j. Cannabinoid Low Ctl Soln (0.05/0.25 µg/mL):

To a 10-mL volumetric flask, add 1.0 mL of the Cannabinoid High Control Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.

k. Negative Control Urine:

Synthetic urine (Surine) may be purchased from Dyna-Tek, Inc., Lenexa, KS; alternatively, blank urine may be obtained in-house. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control Urine Sample is extracted and analyzed with every urine assay.

l. Negative Control Blood:

Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or prepared in-house from an appropriate blank specimen. Store frozen, refrigerated or obtain fresh. Stability determined by manufacturer. In-house Negative Control Blood is stable for at least 2 years when frozen. A Negative Control Blood sample is extracted and analyzed with every blood assay.

m. System Suitability Mixture (0.05/0.25 µg/mL):

Mix 0.1 mL of the High Ctl Solution in an autosampler vial with 0.45 mL water and 0.45 mL acetonitrile. Store refrigerated or in cooled autosampler tray. Stable for at least one week.

7 Sampling

Representative portions of the specimens are obtained. See TOX101 for further details.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the authorized individual performing the procedure.

- a. Add 0.5 mL blood and/or urine to a labeled test tube.
- b. Prepare positive control blood samples:
Low Ctl (3/15 ng/mL): Add 30 μ L Low Ctl Solution to 0.5 mL Negative Control blood.
High Ctl (33/165 ng/mL): Add 33 μ L High Ctl Solution to 0.5 mL Negative Control blood.
- c. Prepare positive control urine samples:
Low Control (20 ng/mL): Add 40 μ L Low Ctl Solution to 0.5 mL Negative Control Urine.
High Control (200 ng/mL): Add 40 μ L High Ctl Solution to 0.5 mL Negative Control Urine.
- d. Add 20 μ L of the THC IS Working Solution to each sample, vortex and allow samples to stand for 15 minutes.

e. For blood samples: Add 2.0 mL cold acetonitrile drop-wise to each blood sample while vortexing. Vortex for ~30s and centrifuge at approximately 3000 rpm for 3 minutes. Remove the supernatant and transfer to a labeled test tube. Add 4 mL deionized water and vortex.

For urine samples: Add 0.075 mL 11.8 N potassium hydroxide to each urine sample. Place on a heating block at approximately 60°C for approximately 15 minutes. Remove from heating block and cool for approximately 5 minutes. Add 0.075 mL glacial acetic acid and vortex. Add 5.0 mL 0.1 M sodium acetate buffer and vortex. Verify pH to be 4.5 - 6.5. If not, adjust with more glacial acetic acid or 11.8 N potassium hydroxide.

- f. Load samples onto CEREX THC solid phase extraction columns and push through with positive pressure.
- g. Wash each column with 1.0 mL Water:Acetonitrile:Ammonia (90:10:1).
- h. Dry each column for 15 minutes under full pressure.
- i. Elute with 2.0 mL Ethyl Acetate by gravity.
- j. Dry each column for 10 minutes at 40°C.
- k. Elute with 2.0 mL Hexane:Ethyl Acetate:Acetic Acid (88:10:2) by gravity into the same tube as above.

- l. Evaporate to dryness at 40°C.
- m. To the evaporated sample, add 0.050 mL acetonitrile, vortex, and transfer to labeled autosampler vial.
- n. To the same evaporated sample, add 0.050 mL water, vortex and transfer to the same autosampler vial.
- o. Add 0.050 mL deionized water to each sample before analysis by LC/MS/MS.
- p. Verify that instrument is operating properly by analyzing the System Suitability Mixture and verifying that all three analytes are present. Analyze samples.

9 Instrumental Conditions

Following are the operating parameters for the instruments used in this procedure. Appendix 2 contains an abbreviated version of instrumental parameters used in this procedure. This checklist may be used by the authorized individual performing the procedure.

9.1 Autosampler Procedures

Autosampler Temperature Setting: 15°C

Injection Volume: 25 µL

9.2 Liquid Chromatograph Parameters (25°C)

Time (min)	Total Flow (mL/min)	%A (0.1% Formic Acid in Water)	%B (0.1% Formic Acid in Acetonitrile)
0.01	0.5	60	40
7.00	0.5	0	100
10.00	0.5	0	100
12.00	0.5	60	40
19.90	0.5	60	40

9.3 Mass Spectrometer Parameters

Scan Mode	Turbo Spray	Polarity	Positive
Resolution	Unit	Scan Type	MRM
Curtain Gas	Nitrogen (35)	Ionspray Voltage	5000
Source Temperature	670°C	Nebulizer Gas	Nitrogen (55)
Entrance Potential	10	Turbo Gas	Nitrogen (55)

Q1 Mass	Q3 Mass	Dwell Time (ms)	Declustering Potential	Collision Energy	Collision Exit Potential
315.143	193.10	25	91	31	12
315.143	123.00	25	91	41	14
315.143	259.10	25	91	27	16
315.143	135.10	25	91	27	10
318.148	196.10	25	106	31	12
318.148	123.00	25	106	39	14
318.148	135.10	25	106	27	14
331.137	193.00	25	81	21	34
331.137	175.00	25	81	33	14
331.137	201.00	25	81	35	20
331.137	105.00	25	81	49	14
334.157	196.10	25	106	33	12
334.157	201.00	25	106	33	12
334.157	175.10	25	106	31	12
345.119	229.10	25	121	27	20
345.119	193.10	25	121	35	12
345.119	187.00	25	121	37	18
345.119	119.144	25	121	35	8
348.171	302.10	25	81	27	18
348.171	196.10	25	81	37	10
348.171	119.10	25	81	35	14

Only the 345 and 348 transitions are routinely collected for the analysis of urine samples.

10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to identify THC or related compounds within a biological specimen:

10.1 Chromatography

All four ion transition peaks for the analyte of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. For low concentrations of analyte (less than 5 ng/mL), there may only be three strong transitions. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.1.1 Retention Time

The retention time of the peak should be within $\pm 2\%$ of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

10.2 Mass Spectrometry

Four independent MS/MS experiments are conducted for each analyte. At least two ion ratios are calculated for each analyte; the ion ratios should compare favorably to ions ratios from a reference standard or an extracted positive control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (TOX104) for further guidance.

11 Calculations

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Limitations

	THC	THC-COOH		THC-OH
	Blood	Blood	Urine	Blood
Limits of Detection (ng/mL):	1	2.5	5	1.0

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

FBI Laboratory Safety Manual.

Quality Control for Toxicology Examinations (TOX101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

Guidelines for Comparison of Mass Spectra (TOX104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Rev #	Issue Date	History
6	09/15/2020	8, 9 - Changed to authorized individual 11, 15 - Update TOX101 title 9.1, Bench notes C17 - Updated injection volume from 10 to 25 μ L
7	12/15/2020	2 - Changed scope to qualitative only 5-b, w - Removed "calibrated" from pipette, updated instrument 6 - Manufacturer clarifications 7 - Added clarifying language for sampling considerations 8 - Deleted quantitative steps 9.2, 9.3 - Updated gradient and mass spectrometer tables 10.2 - Deleted Table 2 (duplicative) 11, 12, 13 - Removed text (quantitative references) 15 - Removed CUQA 13 reference Bench Notes - Updated to reflect above changes.

Approval

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Chemistry Unit - Toxicology
Technical Leader: _____

Date: 12/14/2020

Chemistry Unit Chief: _____

Date: 12/14/2020

Appendix 1: Abbreviated version of the Cannabinoid Procedure for bench use.

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Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.

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